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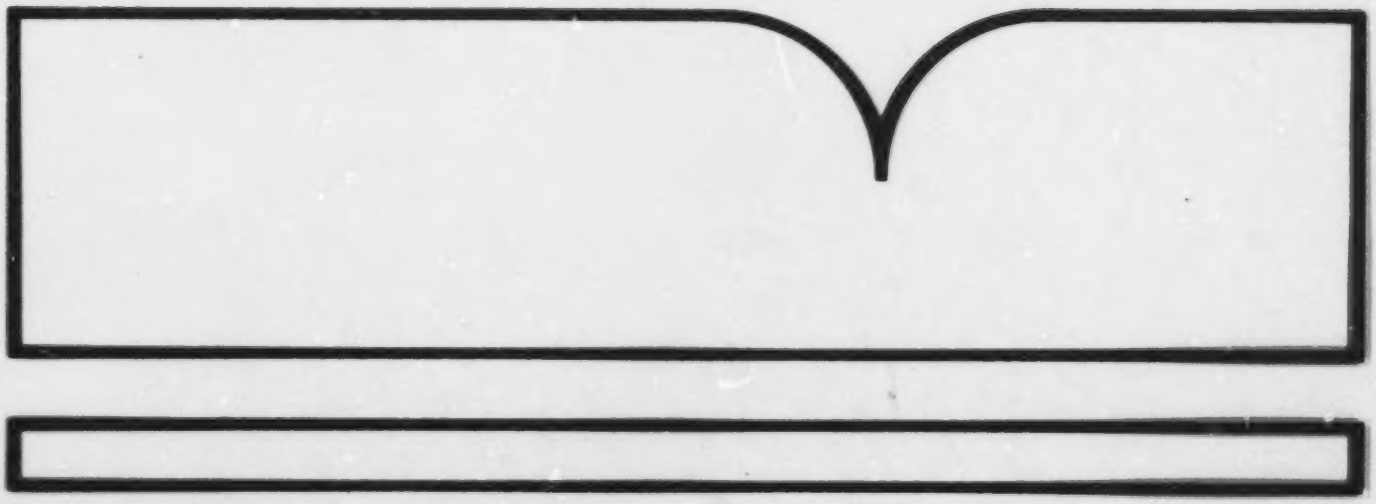

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16. ABSTRACT

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Regardless of occupational exposure, smokers had considerably higher urinary mutagenic activity than non-smokers on Strain TA1538 withS-9, and to a lesser extent on Strain TA100 with S-9.

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Detection of occupational exposure to genotoxic agents with a urinary mutagen assay

David Kriebel [a], Barry Commoner [a], Donald Bollinger [b], Alice Bronsdon [b], Jane Gold [b] and Joyce Henry [b]

[a] *Center for the Biology of Natural Systems, Queens College, CUNY, Flushing, NY 11367 and* [b] *Washington University, St. Louis, MO (U.S.A.)*

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Summary

The levels of mutagenic activity in the urine of 198 smoking and non-smoking chemical workers, coke plant workers and controls were measured using a urinary mutagen assay based on the Ames Salmonella/microsome test. Urine samples were concentrated using the non-polar resin XAD-2 eluted with methylene chloride and acetone. Activity towards Salmonella strains TA1538 and TA100 with and without microsomal preparation S9 was measured. Urinary mutagenic activity was calculated from linear regression models fitted to the data from the methylene chloride and acetone extracts. Both the non-smoking chemical workers and non-smoking coke plant workers had significantly higher mutagenic activity on strain TA1538 with S9 than did a group of office and laboratory worker controls. However, urinary mutagenic activity from non-smoking chemical and coke plant workers on both strains TA1538 and TA100 without S9, and on strain TA100 with S9, was not significantly different from the mutagenic activity of this control group. Regardless of occupational exposure, smokers had considerably higher urinary mutagenic activity than non-smokers on strain TA1538 with S9, and to a lesser extent on strain TA100 with S9. Estimated tar exposure, calculated from reported cigarette consumption rate and reported cigarette brand, was closely correlated with urinary mutagenic activity on strain TA1538 with S9 ($r = 0.73$, $P < 0.001$ in the Spearman rank order correlation test). D-Glucaric acid concentrations were determined in 150 of the urine samples as a measure of mixed-function oxydase activity. No significant differences were found between the exposed and control groups or between smokers and non-smokers.

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I. Introduction

Body fluids, and particularly urine, have been assayed for genotoxicity for at least 10 years [17,18]. The discovery by Yamasaki and Ames [24] in 1977 that smokers' urine was mutagenic in the Ames test prompted a number of studies of the mutagenic activity of populations exposed to various environmental genotoxins [4–7,12,15,22,23]. While several of these studies were negative, as a group they demonstrate that cigarette smoking and exposure to certain occupations do result in elevated levels of mutagenic activity. These findings suggest that a urinary mutagen assay may be useful as a rapid method of detecting exposure to mutagens in groups at risk for such exposures. In this study we identified two populations with potential exposure to mutagens and sampled them for urinary mutagenic activity. Our purpose was to examine the feasibility of a large-scale screening program for populations with diverse, unknown mutagen exposures. New methods of urine concentrate preparation and dose response curve quantification were also tested.

We also assayed the urines for D-glucaric acid concentration. D-Glucaric acid has been reported to be useful as a measure of hepatic mixed-function oxidase (mfo) activity [9–11,16]. However, some inconsistent results have been reported [8,13,20]. We hypothesized that since many environmental mutagens require enzyme-mediated activation, there might be a relationship between body fluid mutagen levels and mfo activity.

II. Methods

The populations

The study populations consisted of workers at two chemical plants and two coke oven plants. Controls were drawn from several sources. Both exposed workers and controls were residents of moderate-sized cities in the central United States. Most were office and laboratory workers at a large university. laboratory workers were not known to have regular or heavy exposure to any mutagens, although some did have occasional contact with certain mutagenic chemicals. Additional control samples came from workers disabled by physical injuries, who had been out of work for at least two weeks, and from family members of workers not occupationally exposed to potential mutagens. Urine samples and questionnaires covering smoking habits, occupation, medications and illnesses were collected from a total of 198 individuals. Urine samples were collected for a period beginning after work and ending at bedtime,and again upon arising, until 800 ml were collected. Between collections, the samples were to be refrigerated or frozen. In many cases. sample bottles were returned with less than 800 ml of urine; as a result, not all of the planned analyses were run on all samples.

Urine samples were collected from 101 chemical workers from two different plants in the Midwest. Neither plant was known to use or manufacture significant quantities of genotoxins. They were diversified plants producing pharmaceuticals,

pesticides and various primary organics for further manufacture. In the larger of the two plants, most production workers operated from ventilated control booths. Maintenance workers reported more significant (but erratic) exposures to chemicals on the job. Both maintenance workers and line operators were sampled; all but 4 were males. 43 coke plant workers were sampled, from two different coke plants in the Midwest. The subjects all had regular duties on or alongside the coke batteries, although not all were actually battery workers. Some mechanics, electricians and maintenance workers were included in the sample. There were two women in the sample. There were 54 individuals in the control group, 12 of whom were women.

The groups were further subdivided into cigarette smokers and non-smokers. (Pipe and cigar smokers were not studied.) On the basis of reported cigarette brand and reported cigarette smoking rate (the number smoked in the previous 24 h), the estimated tar exposure of each smoker was calculated [2]. The number of smokers and non-smokers in each population are: chemical workers — 51 smokers, 50 non-smokers; coke plant workers — 28 smokers, 15 non-smokers; controls — 11 smokers, 43 non-smokers.

Urine mutagenic activity analysis

Urine samples were stored at −25°C until analyzed. The steps followed in analyzing each sample are outlined below.

(1) Each urine sample was thawed, mixed, and the pH and specific density were determined.

(2) A 1-ml aliquot was removed from each sample for creatinine assay (according to the method described in Sigma Technical Bulletin No. 555), and two 11-ml aliquots were removed for D-glucaric acid assay.

(3) The sample was adjusted to pH 6.0 by adding 1 M phosphate buffer and titrating with 6 N HCl or 6 N NaOH. Then the sample was vacuum-filtered through Whatman No. 1 paper.

(4) The sample was divided into two 400-ml portions and passed through two columns each containing an 8 × 0.7 cm bed of XAD-2 resin and connected to an upper glass reservoir. The gravity-flow rate was adjusted to 2–3 ml per minute.

(5) The urine that drained through the column was discarded. 10 ml of methylene chloride were then added to the resin column and allowed to drain through it. The aqueous layer (residual urine) above the methylene chloride extract was removed.

(6) 10 ml of acetone were then placed on the column and drained into a separate tube.

(7) The methylene chloride and acetone eluates were evaporated in the collection tubes at 40°C under a nitrogen manifold. The residues were taken up in 2 ml of dimethyl sulfoxide (DMSO) for testing in the Ames assay.

(8) Materials for the Salmonella/microsome mutagen assay were prepared according to Ames [1]. From each DMSO solution (derived from 400 ml of urine), duplicate aliquots representing 5, 10, 25 and 50 ml of urine were added to tubes of top agar. When sufficient sample was available, samples were tested on both strain TA1538, a detector of frame-shift mutagens, and strain TA100, a detector of base-pair substitution and frameshift mutagens [1], with and without the S9 micro-

somal fraction (prepared from aroclor-1254-induced rat livers) *. The tester strain/S9 combination was added to the top agar, which was poured on plates containing minimal media agar. The plates were incubated at 37°C for 48 h. Each plate was counted for revertant colonies, and the results used to plot separate dose–response curves for the methylene chloride and acetone extracts.

Urinary glucaric acid analysis

The analysis for glucaric acid was run on an 11-ml aliquot of each urine sample, in accordance with Marsh's procedure, as modified by Simmons et al. [19]. The method proceeds in two stages. In the first, D-glucaric acid is converted to glucaro-1,4-lactone by heating at low pH. Glucaro-1,4-lactone is a powerful inhibitor of β-glucuronidase. In the second step, both β-glucuronidase and phenolphthalein glucuronide are added to the samples, which are incubated at 37°C for 40 min. The extent of inhibition of the cleavage of the phenolphthalein glucuronide by β-glucuronidase can be used to calculate glucaric acid concentration in the sample, after the effect of other urinary β-glucuronidase inhibitors has been removed. This latter step is accomplished by running a simultaneous urine sample through the procedure at alkaline, rather than acidic, pH. The major steps in the method are as follows:

(1) Two 11-ml aliquots were removed from each urine sample and stored at −25°C until analysis.

(2) Two 5-ml aliquots were removed from one of the 11-ml samples, and one was adjusted to pH 3.6 and the other to pH 8.0.

(3) Both fractions were heated in a boiling water bath for 60 min.

(4) Both fractions were adjusted to pH 4.8 with formate and Tris buffers and to a volume of 10 ml, and 1-ml aliquots of each were transferred to clean tubes in duplicate.

(5) Phenolphthalein glucuronide and β-glucuronidase were added to each tube, and the tubes were incubated at 37°C for 40 min.

(6) The reaction was stopped by addition of pH 12 glycerine buffer, and the absorbance was read at 555 nm. Standards were run through the acidic side of the procedure to construct a standard curve from which D-glucaric acid concentration was determined. Appropriate blanks were run to correct for endogenous colored matter in the urine.

(7) The data were expressed as micrograms of glucaric acid per mg creatinine, to correct for differences in daily urine volume [19].

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* Of the various tester strains available, only two could beused because of the amounts of urine available for assay. We chose to use strains TA100 and TA1538 because they (and TA98, which is very similar to TA1538) have been shown to be the most sensitive base-pair substitution and frame-shift detecting strains respectively in tests of pure compounds [14]. We elected to use TA1538 rather than the similar but more widely-used TA98 because of the extensive experience of our laboratory with TA1538, which predates the availability of TA98.

Quantification of the level of mutagenicity in urine

A variety of procedures have been used for quantifying the level of mutagenicity of a sample from the dose–response curve which results from the Ames assay. In this study the task was complicated because for each sample there were two resulting curves — one from the methylene chloride extract, and one from the acetone extract. Preliminary analyses of the data from the first 50 samples [3] showed that mutagenic activity frequently appeared in either or both extract, with *most* of the activity generally, but not always, occurring in the methylene chloride extract *. Under these circumstances, we decided to devise a measure of mutagenic activity which incorporates activity occurring in both extracts. The procedure follows:

(1) At each dose of urine tested, the average (of duplicate plates) number of revertant colonies per plate produced by the methylene chloride extract is added to the average number of revertant colonies per plate *at the same dose* produced by the acetone extract. The result is a 'synthetic' dose–response curve of the summed activity of the two extracts (see Fig. 1).

(2) This procedure is performed for doses of urine ranging from 0 to 50 ml of urine per plate.

(3) A simple linear regression is performed to find the best-fit line for the summed dose-response curve between 0 and 50 ml of urine per plate.

(4) The '*b*' value, or slope, of the regression equation is a measure of the steepness of the dose–response curve, and is a direct estimate of the number of revertant colonies per plate per ml of urine tested. The number of spontaneous revertants has, in effect, been subtracted by this procedure.

(5) Approx. 18% of the dose–response curves yielded slopes that were negative and significantly different from zero. These consistently falling curves were not included in the analysis, as a negative value for mutagenic activity has no real meaning. This effect is apparently due to bacteriostatic agents extracted from the urine.

This procedure was performed on nearly 500 summed dose–response curves that resulted from the analysis of 198 samples of one or both strains of Salmonella with and without S9. Fig. 1 shows 4 examples of such dose–response curves, the derived synthetic curve and least-squares line for the summed data. Throughout this paper, data on the mutagenic activity of the urine samples in terms of revertant colonies per plate per ml of urine will be presented. In all cases these data will have been derived from the quantification procedures described above.

The raw data from plate counts were entered directly into a computer data base by lab technicians. After suitable checks for errors, the least-squares regression equation for each composite dose–response curve was calculated, and the relevant coefficients entered into a second computer data base which included all of the

* The appearance of activity in one extract or the other was not related to occupational exposure. In approximately one-third of the samples, activity appeared in the methylene chloride extract, but not in the acetone extract. In another third, the acetone but not the methylene chloride extract was active, and in the final third, activity occurred in both extracts. When activity occurred in both extracts, most of it was in the methylene chloride extract.

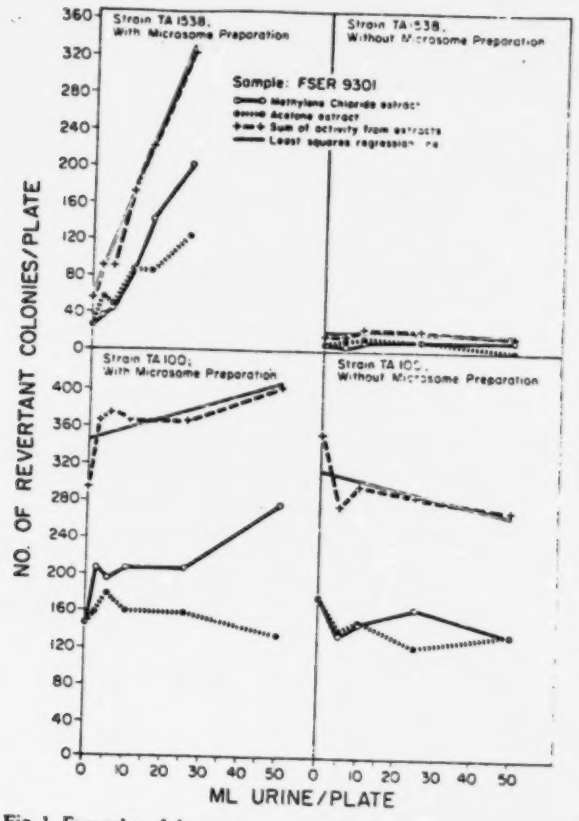

Fig. 1. Examples of dose–response curves of urinary mutagenic activity.

collected information on each subject. This second data base was then used to perform statistical analyses.

III. Results

Urinary mutagenic activity

Not all samples were tested on both strains TA1538 and TA100 with and without S9 because of inadequate amounts of sample, as previously described *. Table 1

* Another reason for the limited number of samples tested on both strains is that initially many samples were plated at urine equivalent volumes out to 100 ml of urine per plate, rather than just out to 50 ml as described in the methods. We have shown elsewhere that plating this very large volume did not improve the sensitivity of the method, but it doubled the volume of urine required and thus limited the number of strains that could be used.

TABLE 1

SAMPLE SIZE FOR EACH STRAIN/S9 COMBINATION BY EXPOSURE GROUP

Group	Number of observations			
	TA100		TA1538	
	With S9	Without S9	With S9	Without S9
Non-smoking controls	39	14	35	7
Non-smoking chemical workers	46	6	47	10
Non-smoking coke plant workers	12	3	12	5
Smoking controls	5	2	10	5
Smoking chemical workers	44	19	50	32
Smoking coke plant workers	21	19	23	25
Total	167	63	177	84

TABLE 2

MUTAGENIC ACTIVITY AND STATISTICAL SIGNIFICANCE BY GROUP

Group	Median value (revertant colonies per plate per ml)				Wilcoxon's Z and (P) [b] for comparison to non-smoking controls			
	TA100		TA1538		TA100		TA1538	
	With S9	Without S9	With S9	Without S9	With S9	Without S9	With S9	Without S9
Non-smoking controls	0.21	0.02	0.03	0.05	–	–	–	–
Non-smoking chemical workers	0.55	0.97 [a]	0.26	0.03	1.44 (0.07)	1.69 (0.05)	1.87 (0.03)	0.10 (0.46)
Non-smoking coke plant workers	0.40	0 [a]	0.68	0.01 [a]	0.73 (0.23)	0.44 (0.33)	2.17 (0.02)	0.49 (0.31)
Smoking controls	0.76 [a]	0.10 [a]	2.03	0.05 [a]	1.35 (0.09)	0.16 (0.44)	3.39 (< 0.01)	0.65 (0.26)
Smoking chemical workers	1.14	0.07	2.60	0	NA [c,d]		NA	
Smoking coke plant workers	0.75	0	2.97	0.07	NA [d]		NA	
Entire population	0.56	0	0.81	0.04	NA		NA	

[a] Fewer than 10 observations; interpret with caution.
[b] A 'P' of < 0.05 was considered statistically significant (one sided test).
[c] Not applicable.
[d] Smoking chemical and coke plant workers were not compared to smoking controls because they differ substantially in tar intake. Thus, a comparison would be testing a combination of the effect of smoking and of the occupation, and the two effects could not be separated.

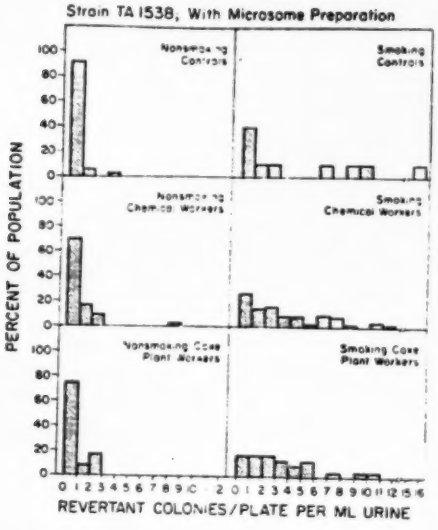

Fig. 2. Urinary mutagenic activity towards strain TA1538.

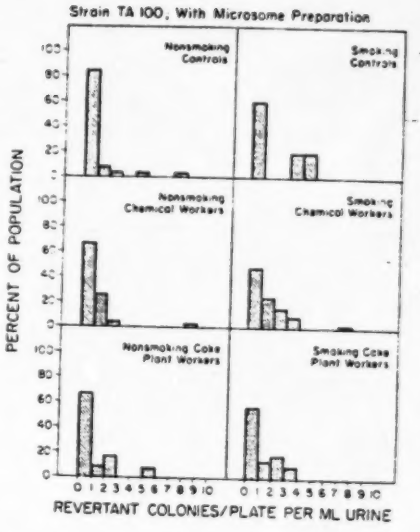

Fig. 3. Urinary mutagenic activity towards strain TA100.

shows the number of observations on each strain/S-9 combination. In Table 2 the mutagenic activity data for all smoking and occupation groups are summarized. The median rather than the mean was used as the summary statistic for presenting the data because the mean is more sensitive to a few very high values (see Fig. 2) in a non-normally distributed data set such as this. Figs. 2 and 3 show the frequency distributions of the data for TA1538 and TA100 with S9. The 'without S9' data are not shown because the number of observations in any exposure group was very limited (see Table 1).

It is clear from Table 2 and Figs. 2 and 3 that the effect of smoking on urinary mutagenic activity was substantial, when tested on strain TA1538 with S9 and more modest but still noticeable on strain TA100 with S9. On either strain without S9, no clear smoking effect is detectable.

The effect of smoking on urinary mutagenic activity was also tested another way. The estimated tar exposure of each person was calculated from reported cigarette smoking rate and reported cigarette brand, as already described. Non-smokers were assigned an estimated tar exposure of zero. A Spearman rank-order correlation coefficient [21] was calculated for the relationship between estimated tar exposure and mutagenic activity towards both strains with and without S9 for all individuals, regardless of occupational exposure. The results are shown in Table 3. For activity towards TA1538 with S9, estimated tar exposure has a strong effect on urinary

TABLE 3

SPEARMAN RANK-ORDER CORRELATION COEFFICIENTS FOR TAR INTAKE vs. MUTAGENIC ACTIVITY

	TA100 without S9	TA100 with S9	TA1538 without S9	TA1538 with S9
Correlation coefficient (r)	−0.12	0.28 *	0.10	0.73 **
Number of samples (n)	46	121	51	120

* $P < 0.01$.
** $P < 0.001$.

mutagenic activity, regardless of occupational exposure. The correlation is smaller but still significant when TA100 with S9 is used.

Several statistical tests were performed to determine whether chemical workers or coke oven workers had higher levels of urinary mutagenic activity than did controls. Inspection of Table 2 suggests that such an effect may occur in the data from testing with TA1538 with S9, and possibly also from TA100 with S9. The data without S9 in Table 2 seem quite erratic, possibly due to small sample sizes. But because smoking has no effect on either strain when urine samples are plated without S9, the smoking and non-smoking groups can be combined. When this is done, neither occupational group has significantly higher urinary mutagenic activity than the control group towards either strain of Salmonella, without S9 (revertant colonies per plate per ml of urine for controls, chemical workers and coke oven workers respectively: TA1538 — 0.05, 0.00, 0.07; TA100 — 0.02; 0.24, 0.00) *.

Comparing the occupational groups for activity towards either strain with S9 is made difficult by the strong smoking effects. Groups of smokers with different occupational exposures cannot be directly compared unless it can be shown that each group has identical tar exposures. Non-smokers with different occupational exposures can, however, be compared, and Table 2 shows that there are clear differences amongst non-smoking groups on TA1538 with S9 and to a lesser extent on TA100 with S9. The median mutagenic activity towards TA1538 with S9 of control non-smokers was 0.03 revertant colonies per plate per ml of urine. For non-smoking chemical workers, the median was 0.26; and for non-smoking coke oven workers, it was 0.68 revertant colonies per plate per ml of urine. In the Wilcoxon rank-sum test [21], a standard non-parametric test for comparing groups (analogous to the t-test for parametric data), the non-smoking chemical workers had significantly higher urinary mutagenic activity than non-smoking controls (see Table 2). In the same statistical test, non-smoking coke oven workers also had significantly higher activity than non-smoking controls. Corresponding comparisons

* The Wilcoxon rank-sum test was used for these comparisons.

TABLE 4

CREATININE AND GLUCARIC ACID DATA BY GROUP

Group	*N*	Unadjusted glucaric acid concentration (μM)	Creatinine (mg/100 ml)	Adjusted glucaric acid concentration (μg/mg creatinine)
Non-smoking controls	33	22.1	111	4.18
Non-smoking chemical workers	38	24.4	129	3.96
Non-smoking coke plant workers	11	25.0	179 [a]	2.93 [a]
Smoking controls	6	26.1	130	4.21
Smoking chemical workers	40	22.8	109	4.41
Smoking coke plant workers	22	27.1	149	3.82

[a] *t*-test significant at $P < 0.05$ in comparison to non-smoking controls.

of the data from testing with TA100 with S9 did not reveal statistically significant differences.

Glucaric acid assay results

Glucaric acid results are available on 150 samples. Unlike the mutagenicity data, these data were approximately normally distributed, so *t*-tests were performed to test for differences amongst groups. The data are summarized in Table 4. The mean value for non-smoking coke plant workers is significantly lower than the mean value for non-smoking controls (2.93 μg/mg creatinine for non-smoking coke plant workers versus 4.18 μg/mg for non-smoking controls — significant in a two-tailed *t*-test at $P < 0.05$). The difference between means for the smoking groups is not significant, nor are non-smoking chemical workers different from controls, for mean adjusted glucaric acid. When all smokers are compared to all non-smokers in a *t*-test, the difference in mean glucaric acid concentration is not significant. When the creatinine data and glucaric acid data (unadjusted by the level of creatinine) are examined (Table 4), the possibility arises that the single significant difference between groups, for adjusted glucaric acid, is due to a difference between mean creatinine levels for the two groups. The possibility of creatinine confounding the glucaric acid data is discussed below.

Several statistical methods were used to investigate the hypothesis that there might be glucaric acid/urinary mutagen interactions; however, no evidence of an interaction with activity towards either strain was noted.

IV. Discussion

Our groups of both chemical workers and coke oven workers did have modestly but significantly higher levels of mutagenic activity in their urine than did a group of control workers.

It must be emphasized that these differences exist for the *groups*, and that the individual values varied substantially. This caution can best be underscored by referring to Figs. 2 and 3. It is clear that the ranges of values for all populations overlap, and that what causes the median for one group of data to be higher than another is a few very high values or the relative absence of very low values.

Although our occupational populatons were drawn from industries with known carcinogenic hazards, the plants that we sampled from were not known to have serious carcinogen or mutagen exposure problems *. Thus, we regard our test populations as 'potentially exposed' rather than 'certainly exposed.' We note here that Dolara et al. [4] used nearly identical testing methods and found considerably larger differences between a control and chemical worker group, but there was much firmer evidence of carcinogen exposure in their plants.

Smoking was clearly the major determinant of urinary mutagen activity in this study population, which does not necessarily mean that this is the major source of environmental mutagens, but in this test, in these populations, smoking does dominate. Even without controlling for occupation effects, urinary mutagenic activity was very well correlated with estimated tar exposure ($r = 0.73$ in the Spearman rank order correlation). This finding (and similar results by Van Doorn et al. [23]) represents the best evidence to date that there is a dose–response relationship between the level of exposure to a mutagen and the concentration of mutagens in the urine. It is significant that this occurs despite the crude nature of the measure of exposure and the fact that the urinary mutagenic activity is unadjusted for differences in daily urine volume. (In using a urine sample derived from much less than a 24-h period, we are measuring the *concentration* of mutagens in the urine. The *amount* of mutagens excreted would be more relevant, but this requires a 24-h sample, which can be difficult to obtain from working populations, particularly on round-the-clock shifts.)

This study has shown that higher levels of mutagens can be detected in the urine of populations with potential but unknown occupational exposure to mutagens, than in control populations. In the absence of chemical identification of the mutagens, this observation does not in itself establish that the elevated activity observed in urine from the occupational groups is due to substances that occur in the workplace. Conceivably, some non-occupational behavioral pattern associated with these groups might account for the results. It is also important to remember that the cases and controls differed in many potentially important ways besides mutagen exposure. The cases (particularly coke plant workers) worked in hot environments and were engaged in considerable physical activity, while the controls worked in generally sedentary jobs in temperature-controlled workplaces. Our study also suggests that cigarette smoking is a major source of mutagens in occupational populations and that it significantly interferes with attempts to identify other sources of urinary

* In fact, they were probably cleaner than average for their industries. As mentioned previously, the larger chemical plant was well automated, and operators worked from enclosed booths. Both coke ovens were less than 5 years old.

mutagens. If preliminary tests show that a particular exposure can be detected with a strain that is not sensitive to mutagens derived from tobacco smoke, this will simplify the study considerably. Examples of such strains are Salmonella TA1535 and TA1537 [24] and E. coli WP2 [7].

Glucaric acid as a measure of enzyme activity

Several studies in the literature have suggested that glucaric acid levels in urine may be used as a measure of mixed function oxidase activity. One paper [16] reported differences in glucaric acid levels between workers exposed to insecticides and other organic chemicals, and a control group. In this study of chemical and coke plant workers, no differences in glucaric acid excretion among occupational groups were observed. The one statistically significant difference in a test of means (between non-smoking coke plant workers and non-smoking controls) is most likely due to confounding factors. Creatinine concentrations (used in adjusting glucaric acid values) were significantly elevated in the coke plant workers (see Table 4), and this explains the low level of adjusted glucaric acid found in this group. Creatinine concentrations in urine are related to muscle mass (positively), liquid intake (negatively), and physical exertion (positively). It would not be surprising to find that coke plant workers differ from the control group (mostly laboratory and office workers) in some of these variables.

Because of the possibility of inter-occupational differences in urinary creatinine levels, it may be difficult to use glucaric acid data in studies like this one. In a protocol in which subjects served as their own controls (for example, before and then during an exposure), these confounding factors might be largely eliminated, and so the glucaric acid assay might be useful. In addition, some improvements in the assay itself would be desirable. Variations between repeated runs on the same urine were rather large compared to variations between repeated determinations of unknown potassium glucarate water solutions.

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